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Use of Salmon Cardiac Primary Cultures (SCPCs) of different genotypes for comparative kinetics of *mx* expression, viral load and ultrastructure pathology, after infection with S*almon Pancreas Disease Virus* (SPDV).

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Abstract

In vitro fish based models have been extensively applied in human biomedical research but, paradoxically, less frequently in the research of fish health issues. Farmed Atlantic salmon can suffer from several viral conditions affecting the heart. Therefore, species-specific, cardiac *in vitro* models may represent a useful tool to help further understanding and management of these diseases. The mechanisms underlying genotype based resistance are complex and usually rely on a combined effect of elements from both the innate and adaptive immune response, which are further complicated by external environmental factors. Here we propose that Salmon Cardiac Primary Cultures (SCPCs) are a useful tool to investigate these mechanisms as the basis for genotypic differences between Atlantic salmon families in susceptibility to cardiotropic viral disease.

Using SCPCs produced from two different commercially available Atlantic salmon embryonated ova (Atlantic Ova IPN sensitive" (S) and "Atlantic QTL-innOva® IPN/PD" (R)), the influence of host genotype on the viral load and *mx* expression following *Salmon Pancreas Disease Virus* infection was assessed over a 15 day period. Both R and S SCPCs groups were successfully infected. A measurable difference between groups of viral *nsP1* and host antiviral *mx* gene expression was observed (i.e. a later, but larger onset of *mx* expression in the R group). *Mx* expression peaks were followed by a decrease in viral *nsP1* in both groups. Additionally, ultrastructural examination of infected SCPCs allowed the description of degenerative changes at the individual cell level. The SCPC model presents some advantages, over current fish cell culture monolayers and *in vivo* material, such as the presence of different cell components normally present in the target organ, as well as the removal of a layer of functional complexity (acquired immunity), making it possible to focus on tissue specific, early innate immune mechanisms. These preliminary results highlight the importance of considering genetic origin when selecting the fish source for the production of SCPCs, as well as their usefulness as screening tools for assessment of genotypic differences in disease resistance.

1. Introduction

In the last decade an increased number of viral conditions associated with severe cardiomyopathy have impacted farmed salmonids, such as those caused by Piscine Cardiomyopathy Virus (PCMV), Piscine Reo Virus (PRV) and *Salmon Pancreas Disease Virus* (SPDV), also commonly referred to as *Salmonid Alphavirus* (SAV) [1–4].For consistency in this work and given that the first is yet the only accepted species name approved by the International Committee on Taxonomy of Viruses, we will refer to it as SPDV (ICTV Feb. 2013).

The focusing of this work on SPDV arises from the severe economic and animal welfare impact this virus has had for over 30 years, threatening the sustainability of Atlantic salmon aquaculture [5,6]. Variability in the outcome of the SPDV infection is provided by differences in virulence between SPDV subtypes and strains [7]. However, differences in host susceptibility had long been suspected and were eventually confirmed through an epidemiological survey in Ireland, which indicated significant differences between diverse fish stocks, evaluated by levels of clinical disease and mortality. These differences were further confirmed through experimental challenges [8].

The concept of host natural resistance is the basis for development of selection-based approaches to disease control. As is seen with humans and other animals, the wide variation in outcomes of a host pathogen interaction reflects a complex interplay of environmental and host factors. Among the most important intrinsic determinants of resistant/susceptibility to infection are the host genetics, and the functional diversity of immune response [9,10]. For example, differences in the innate antiviral response measured as interferon (IFN) induced *mx* gene expression, were found between different cell lines of salmon origin, correlating with their resistance and susceptibility [11,12].

In vivo and *in vitro* fish models have been extensively used in human biomedical research in a wide range of fields, and notably the zebra fish (*Danio rerio*) is today a strongly established vertebrate model for the study of human disease [13–15]. *In vivo* models are widely used in disease research in production fish. Paradoxically, in comparison the application of *in vitro* fish based models to study disease in production fish is relatively limited, although the potential is high. Traditional *in vitro* work using continuous fish cell lines has historically provided important insights; however it does pose certain limitations and risks. For example, contamination and replacement of the original cell type has been reported in EPC (Epithelioma papulosum cyprini), where currently used cultures were found to be contaminated by fathead minnow (*Pimephales promelas*) cells [16]. There is also the possibility that mechanisms observed *in vitro* are not translatable to the live host, for instance adaptation of a virus after repetitive passage resulting in tissue culture adaptation and consequent loss of virulence in host challenge studies [17–19]. Difference in *in vivo* versus *in vitro* outcomes can also be related to immune cell interactions, giving rise to potential for adaptive immunity in the former versus predominantly innate immune responses in the latter.

While *in vitro* models are a simplified version of the host, they are desirable tools that could help replace or reduce the number of live animals required in experimental work [20,21]. A major drawback is the limited number of cell lines of the target species available. One critical aspect required to translate output from cell line models to animal is the availability of cell lines corresponding to the cell type present in the target organ. Using SCPCs it is possible to introduce several desirable factors that contribute to its translatability. Two such factors are increased complexity by inclusion of a mixture of cell types mimicking those present in the target organ, and isolation from the host species to which the model applies. For instance, working directly with rainbow trout tissues proved relevant when studying Sleeping Disease (SD), a condition in freshwater rainbow trout (*Oncorhynchus mykiss*) caused by a SPDV subtype. The use of primary cultures of muscle satellite cells from rainbow trout provided novel evidence that these cells were targeted during infection [22]. In the above scenario, development of *in vitro*

salmonid cardiac models for the study of the mechanisms underlying cardiotropic viral diseases of salmon represents an area of interest.

We developed an *in vitro* 3D heart primary culture model, the salmon cardiac primary culture (SCPC), from embryonic Atlantic salmon (*Salmo salar*). SCPCs contain several of the basic components of the fish heart, *i.e.* myocardium, epicardium, endocardium and fibroblast cells, and can be kept up to 5 months in culture with low maintenance, can be processed for histology, electron microscopy, and molecular techniques, and are permissive to viral infection and replication [23].

We hypothesized that the origin of salmon eggs used to generate the SCPCs can result in differences in the susceptibility of the SCPC model to SPDV. To assess this, we compared the kinetics of infection with a virulent SPDV subtype 1 isolate (SPDV) between SCPCs isolated from embryos of two different, commercially available Atlantic salmon stocks with different genetic traits. The possibility of assessing potential differences in host response due to genomic variability can widen the SCPCs application in genomic selection studies.

2. Material and Methods

2.1. Embryo origin

Commercially available embryonated eggs “Atlantic Ova IPN sensitive” (S) and “Atlantic QTL-innOva® IPN/PD” (R) were obtained from AquaGen® Norway (through AquaGen Scotland Ltd, Stirling University Innovation Park, Stirling, UK). The former has a genotype sensitive to Infectious Pancreatic Necrosis Virus (IPNV) and is usually sourced for use in vaccine trials. The Atlantic QTL-innOva® IPN/PD is a genotype with a combined increased resistance to both IPNV and to SPDV (pancreas disease – PD), as described by the manufacturer.

2.2. SCPC production

SCPCs were produced following protocols previously described [23]. In brief, eggs from both genotypes, described above, were incubated separately in aerated, sterile sea water at 5ºC with primary isolation and harvesting of the identified cardiac tissue performed over a 2 week period prior to hatching. Embryos were stripped from their shell using sterile tweezers and scissors in a laminar flow cabinet. They were then transferred into 0.5 ml Trypsin (2.5% Trypsin 10x Gibco by Life Technologies-NZ), and mechanically dissociated to facilitate chemical digestion. The lysate was transferred into 2ml of culture medium (L15 (Lonza, UK) supplemented with 10% foetal bovine serum (Sigma-Aldrich, USA), 100 U/ml Penicillin and 0.1 mg/ml Streptomycin (Fisher Scientific, UK)), and then centrifuged for 5 minutes at 565g at 15°C. The medium was then removed and 2ml of fresh medium was added pipetting vigorously to re-suspend the tissue pellet. Each whole embryo lysate was then plated individually into 12 well tissue culture plates (Cellstar-Greiner Bio-one-UK) and incubated at 20°C. Culture medium was then renewed at 24h simultaneously removing the non-attached cell debris. Plates were examined daily under a dissecting microscope (Motic-SMZ 168) to collect developing SCPCs. These were recognised by their morphology and their beating capacity, and where carefully pipetted out using 100 or 200µl tips. SCPCs were

then individually transferred to x8 well chambered glass slides (Nunc® Lab-Tek II, Thermo-Fisher-USA) with 0.5ml fresh culture media where they re-attached spontaneously and were incubated at 20°C. SCPCs for both genotype groups were isolated and cultured in parallel to ensure synchronous development during the experiments.

2.3. Experimental design

SCPCs between 2-3 weeks old were identified as “S” (sensitive) or “R” (resistant) as per genotype of origin, and were challenged with SPDV virus. SPDV-1 isolate F07-220 originating from a field outbreak in Ireland, was used for the challenge. The virus was at passage 4 and was cultured in Chinook salmon (*Oncorhynchus tshawytscha)* cells (CHSE-214, ATCC CRL 1681) for 7 days. Its virulence and pathogenicity had been tested through an *in vivo* challenge study (unpublished data), where it produced moderate to severe PD histopathological changes. SCPCs were infected synchronously with identical viral dose by adding 50µl of a SPDV-1 F07-220 virus stock solution directly into each SCPCs well to achieve a final dilution of $2.4x10^4$ plaque forming units (PFU)/ml. After 2h absorption at 15ºC, the culture media was exchanged by fresh media both in the infected treatment and control wells and incubation continued at 15ºC.
The experiment was run for 2 weeks using a total of 48 individual SCPCs distributed to have x4 infected and x2 controls per time point and genotype. Samples were taken individually at days 1, 10 and 15 days post infection (dpi), where 0dpi was considered the point of medium exchange after a 2h viral absorption for infected and uninfected medium exchange for controls, respectively.

2.4. Quantitative PCR

Extraction of mRNA was performed from individual SCPCs using Dynabeads® mRNA Direct™ Micro Purification Kit (Catalog Nº 61021, Ambion-ThermoFisher Scientific, UK) as per manufacturer’s instructions. The kit is designed to isolate highly purified mRNA directly from crude lysates and is especially suitable for micro-samples. Elution was performed in a final volume of 10µl of elution buffer (10nM Tris-HCl ph 7.5) from which an aliquot of 7.7µl mRNA was used for reverse transcription (RT) using a Taqman Reverse Transcription Reagent Kit (Applied Biosystems-ThermoFisher Scientific, UK) as follows: 7.7µl of mRNA and 1µl of 50µM Oligo $d(T)^{16}$ (Invitrogen®, ThermoFisher Scientific, UK) were mixed and heated to 70 °C for 10 min and then chilled on ice. The final volume was adjusted to 20 µl by adding 11.3µl of Master mix, comprised of 10x RT buffer (25 mM Tris–HCl pH 8.3, 37.5 mM KCl, 5.5 mM MgCl2), 0.5 mM dNTP, 0.4 U RNase inhibitor and 1.25 U Multiscribe Reverse Transcriptase. The reaction was incubated at 48°C for 90 min, heat inactivated at 95°C for 5 min, and stored at -80°C until use. Quantitative PCR (QPCR) assays were performed on a Roche LC480 System (Roche). TaqMan probes and primers to amplify the elongation factor *elf*, *mx* and SPDV non-structural protein P1 (*nsP1*) are given in Gahlawat et al., 2009. Experimental negative controls were routinely included at the stages of extraction, reverse transcription and QPCR.

The Ct (cycle threshold) was determined by the maximum secondary derivative method where the values were converted into expression levels using a standard curve [24]. The relative expression level of target genes (*mx* and *nsP1*) were then normalized to the level of expression of elongation factor 1 alpha (*elf*), allowing for comparison within and

between genotypes. The errors of normalised gene expression are unlikely to be normally distributed and the most appropriate statistical analysis would utilise a permutation test. However, the combined effect of small group sizes and the need to correct for multiple comparisons mean that such analyses are unlikely to generate statistically significant p-values even if substantial biological differences are present. We have represented differences expressed in terms of "fold of increase", providing also both points estimates (means) and estimates of variance (standard deviation). Fold changes were calculated by comparison to the corresponding non infected controls (*mx*) or relative to day 1 post infection (*nsP1*).

2.5. Ultrastructural studies

For ultrastructural analysis of viral tropism, an identical infection protocol was applied using x2 SCPCs per time point/genotype and sampling at 2, 3 and 24hours post infection (hpi). Samples were fixed *in situ* by adding 0.5 ml cold Karnovsky's fixative (2.5% glutaraldehyde and 2% paraformaldehyde in 0.1M phosphate buffer pH 7.4 (Electron Microscopy Sciences, USA) to the well and left for 30 minutes at room temperature. The fixative contained Fixed SCPCs were then transferred to a 1.5 ml Eppendorf tube with 0.1M Phosphate buffer saline pH 7.4 (PBS) and washed for 15 minutes on a rotator disk placed on a rocker. The procedure was repeated twice followed by transfer to fresh buffer for short-term storage at 4°C. The post fixation used 1% OsO_4 in 0.1 M Cacodylate buffer for 2 h at 4°C followed by dehydration using a graded series of ethanol. Samples were then embedded in araldite resin (Fluka, Switzerland) and ultrathin sections cut on an Ultracut E (LEICA, Germany). Staining was performed with 0.5% uranyl acetate (Laurylab, France) and 3% lead citrate (Laurylab) in an Ultrastainer AC20 (LEICA, Austria). Examination of stained sections was performed on/with a JEOL electron microscope JEM 1011 at 60 kV or JEM-1400 Plus at 120kV (JEOL, Japan).

3. Results and Discussion

SCPCs, a species-specific cardiac model developed from A. salmon embryos, has been proposed as an alternative approach to traditional *in vitro* work using cell lines with the aim to support studies of host pathogen interactions of cardiotropic viral agents, such as SPDV affecting A. salmon. The current work used SCPCs isolated from commercial A. salmon strains resistant to PD/IPN (R), and susceptible to IPN (S), as representatives of different genetic traits. While IPNV resistance has been found to represent a rare case where a single major gene or quantitative trait loci (QTL) explains the bulk of variation in resistance due to the genotype [25],the biological basis of genetic resistance to SPDV is more complex (*i.e.* polygenic)[26]. This work constitutes the first assessment of the usefulness of the SCPC model to evaluate the potential influence of the host genotype in the outcome of SPDV infections. SCPCs were infected synchronously with identical viral dose and the QPCR Ct values were normalised against the individual housekeeping gene (*elf*), therefore it is possible to compare the relative expression within and between genotypes.

3.1. Kinetics of mx and SPDV-*nsP1*

Both SCPCs of R or S origin showed an *mx* gene induction after infection with isolate SPDV-1 F07-220 over a 15 day period.

At 1dpi the *mx* expression in the S group showed a 24.7 fold increase relative to its control, compared to only a 1.6 fold increase in the R group. This was followed by a substantial induction of *mx* in the R group by 5dpi that with a magnitude of 5700 fold increase represents its peak at the time the S group with a 7.7-fold increase showed a transient decline compared to day 1. At 10dpi the R group had a 110 fold increase relative to control, a decrease from its peak expression of day 5, which is then maintained at similar values by 15 dpi with a221 fold increase. In the S group the *mx* gene expression at 10 dpi showed a 812-fold increase representing the peak for this group and it also remained at a similar magnitude by 15dpi with a 219-fold increase relative to controls (Fig 1a-b).

The viral transcript (SPDV-*nsP1*) in both groups had similar expression at 1dpi but afterwards differences between R and S groups were observed, both in magnitude and in kinetics. The earliest increase in *nsP1* was noted in the R group at 5dpi (2266-fold relative to day 1) which corresponded to the peak of *mx gene* expression for this group. This was followed by a transient decrease at 10dpi (31-fold) and a second peak of expression at 15dpi, with a 1619-fold increase, reaching similar levels as that of 5dpi.

Conversely in the S group *nsP1* remained similar at the 2 first time points with only a slight increase at 5dpi, (1.4 fold) coinciding with a decreased *mx* expression on that day. This was followed by a delayed but strong increase at 10dpi (289 fold) simultaneously with the increase in *mx* expression. Similar to observations on the the R group, the increased and sustained level of *mx* expression seemed to induce the subsequent marked reduction on viral gene expression observed by 15dpi (5.6 fold) (Fig 1a-b).

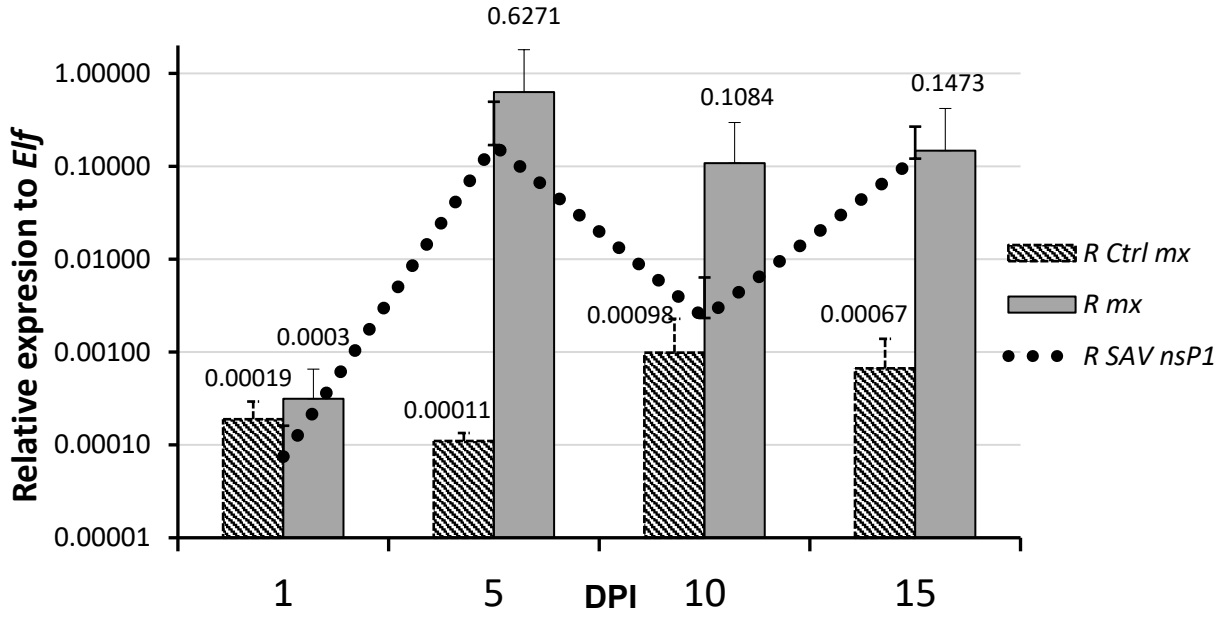

Fig. 1 A: Kinetics of *mx* and SPDV-*nsP1* gene expression in "R" SCPCs genotype after infection with isolate SPDV-1 F07-220. Values are normalised against the host reference gene *elf* and expressed in a logarithmic scale. There are 4 biological replicates/time point. DPI: days post infection.

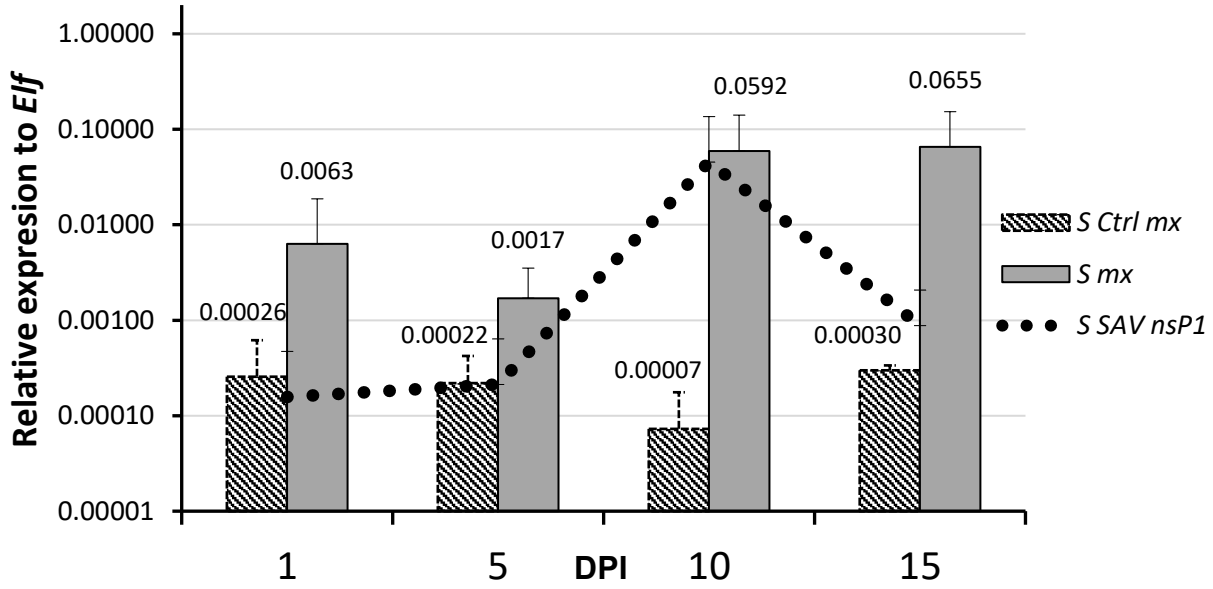

Fig. 1B: Kinetics of *mx* and SPDV-*nsP1* gene expression in "S" SCPCs genotype after infection with isolate SPDV-1 F07-220. Values are normalised against the host reference gene *elf* and expressed in a logarithmic scale. There are 4 biological replicates/time points. DPI: days post infection.

In this study overall the *mx* expression of infected SCPCs after viral induction remained higher than control SCPCs in both groups and at all-time points.
The baseline of *mx* expression on the control groups fluctuated slightly over time. The *mx* average of the controls were 0.00049 (R) and 0.00021(S). Differences in the basal gene expression in the unchallenged SCPCs may have been due to individual variation and given the small sample size in this case, further work is required to verify whether they are determined or not by the origin/genotype of the SCPCs.

Among infected cultures, after induction both groups maintained an *mx* expression level above the base line for the length of the experiment. The S group showed an early *mx* induction followed by a transient decrease before a peak expression for the group at day 10pi, a delayed response compared to the peak on the R group at 5dpi. The magnitude of *mx* expression of the R group was consistently higher from day 5 onwards than in group S, with a general average of 0.2208 and 0.0332, respectively.
Conversely, on average, the total viral RNA expression level was higher in the R group (0.0731) than the S group (0.117), suggesting a relation to the expression of *mx* on each group.

Slow or reduced *mx* gene induction has been observed and associated with a strong viral replication in fish cell lines infected with IPNV [27] and SPDV[19]. Additionally, the amplitude of *mx* gene induction after infection with SPDV was negatively correlated with the viral gene expression in two salmonid cell lines that may have had different levels of resistance to this virus [11,12].
There were fluctuations in the expression of viral genome level in both groups. As experimental settings involved terminal sampling of all replicates at each time point, the expression indicates the cumulative effect and consequently, a peak in viral load at

terminal sampling may represent a re-infection cycle of those samples after the 2 weeks period.

Factors other than the genetic trait itself may have influenced this outcome, including for example the models cell's composition, with a higher level of complexity than standard cell lines. Temperature and the cell line have been reported to influence the culture characteristics of SPDV *in vitro* [28], but these factors were constant in the present study. The genotype R has been commercially selected for SPDV resistance (Aquagen, Norway) and this may explain the relatively overall stronger *mx* response noted in our study when compared with genotype S, which has not been selected for SPDV resistance. These results parallel previous observations.

The kinetics of *mx* expression observed in the R group suggests a possible re-infection event. However if a second viral peak as occurred in the R group, would eventually also happened in the S group over time (after the delay on the peak response) cannot be ruled out with the experimental setting currently used.

3.2. Ultrastructure

Ultrastructural examination confirmed both SCPCs of R and S origin are permissive to infection and viral replication. Qualitative, quantitative or temporal differences in the viral cycle or tropism were not noted between groups with this experimental design (*i.e.* x2 SCPCs samples per group at each of 2, 3 and 24hpi).

All SCPCs were alive and beating at the time of fixation. However, degenerative changes were observed at the individual cell level (Fig 2 A). Changes included mitochondrial swelling and cristolysis (Fig 2 B), cytosol dilution, hydropic degeneration, development of myelin figures and nuclear pyknosis (Fig 2 C-D). Intracytoplasmic viral like particles (Fig 2 D) were observed in cardiomyocytes, endothelial cells, fibroblast and occasional intraluminal leukocytes. Abundant cytoplasmic vacuolar structures such as cytopathic vacuoles, phagosomes and autophagosomes were observed in both cardiomyocyte and endothelial cells (Fig 2 D and F). Sarcomere structural loss was also noted. Endothelial cells seemingly developed degenerative changes earlier (3hpi) than cardiomyocytes (24hpi onwards) and occasionally endothelial cells were seen detaching (Fig 2 F).

Whereas the ultrastructural study did not reveal differences between the R and S groups, it confirmed the permissiveness to infection of both genotypes. It also revealed pathological changes similar to those previously reported during *in vivo* studies. SPDV is known to establish persistent but frequently non-lethal infections, with early histopathological changes described as focalised individual myocardial necrosis [5,29] . Additionally, this work showed the involvement of endothelial cells and a potential role for them in SPDV virus entry. Endothelial cells from liver and brain have been reported as one of the pivotal target cells for viral replication in other alphaviral infections, such as Chikungunya virus (CHIKV) [30] and previous *in vivo* infections in salmon and trout with SPDV have shown infection of cells resembling hypertrophied scavenger endothelium-like cells in the kidney [31], and their potential role in the disease was speculated upon suggesting the current observation of early involvement of cardiac endhotelium in the SPDV infection would deserve further attention.

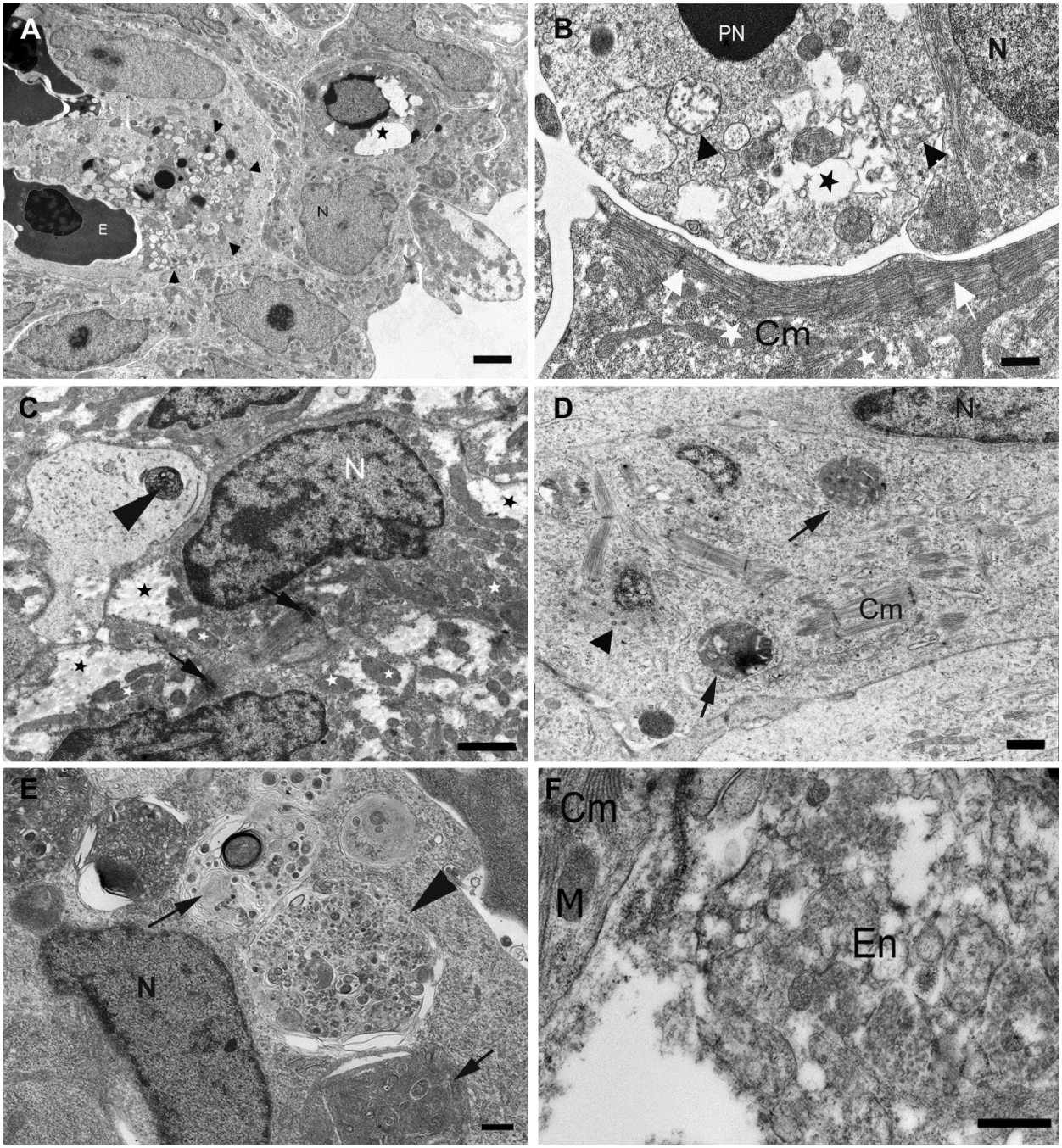

Fig 2: Transmission electron micrograph of SPDV infected Salmon cardiac primary cultures at 3-24hpi. (A). Multifocal, single cell degeneration. A necrotic endothelial cell (black arrow heads) and a single cell with nuclear chromatin margination (white arrow head) and cytoplasmic hydropic degeneration (*) are among intact cells. Bar=2µm. (B). Single cell showing chromatin condensation (pyknosis - PN), swollen mitochondria, cristolysis (black arrowheads) and cytoplasmic hydropic degeneration (stars). Bar=500nm. (C). Early formation of intracytoplasmic myelin figures (autophagosome - arrowhead) in cell with marked cytosol dilution and hydropic degeneration (black stars). Adjacent normal cardiomyocyte shows abundant mitochondria (white stars) and intact intercalated disks (black arrows). Bar=500nm. (D). Dgeneration, sarcomere loss and cytosol dilution in a cardiomyocyte with large cytoplasmic vacuole (arrows) and free virus like particles (arrow heads). Bar 500nm. (E). Large multi-vacuolar structures (arrowhead) containing autophagosomes figures and electron-dense intraluminal vesicles, consistent with autophagolysosomes. Multilamellar bodies with whorls of membranes forming myelin type figures (arrows). Bar=500nm. (F) Abundant cytoplasmic vesicular structures and degenerated mitochondria in a detaching degenerating endothelial cell. Bar= 500nm. N=nucleus, Pn=pyknotic nuclei, Cm cardiomyocyte, E=erythrocyte, En=endothelial cell.

4. Conclusions

This work used SCPCs as representative of different commercially available A. salmon genotypes to assess the potential of the SCPCs as a model to investigate the influence of host origin on the outcome of SPDV infection. Results show that after SPDV infection the two groups display a measurable difference on viral *nsP1* and host antiviral *mx* gene expressions.

The SPCPCs model does not have influence of a systemic, acquired immune response. This can be seen as a missing element therefore a disadvantage, however it could also represent a useful feature as the removal of a layer of functional complexity enables the focus on the study of tissue specific, early antiviral innate immune mechanisms.

Despite the scope of this prospective project was limited and the fact that the mechanisms of genotype based host resistance for SPDV are likely to rely on a combined effect of host and other factors (i.e. not exclusively dependant on *mx* activation), these results indicate that the SCPC model can be used for screening host response differences to viral infection. Moreover, the model showed to be suitable for ultrastructure examination bringing the benefit of the cell component of the heart to be included in the analysis. This would open the opportunity for more comprehensive SPDV viral cycle studies based on the tissue of one of the most affected organs during the disease condition.

With several viral conditions affecting the heart of Atlantic salmon the development of species-specific *in vitro* models represents a step forwards in line with current bioethics standards. Whilst more information is required to in order to establish the SCPCs model as a tool for host genotypic screening, the present results showing measurable differences using the trait of disease resistance are promising. Results have also highlighted that, depending of the scopes, there will be a need to consider the consistency of genetic origin when selecting source of live material for the production of SCPCs. Further work to confirm the robustness of the model should include increasing the sample size, the comparison of using single individual samples versus multiple SCPCs (pooling), and exploring the potential of non-destructive approaches, such as sequential supernatant collection. The generation of assessment panels of biomarkers including other relevant genes, the application of tools such as RNAseq and microarray hybridization as well as immunocytochemistry ad live cell imaging, could further enrich the applicability of the model.

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